# Transient Expression of Epidermal Filaggrin in Cultured Cells Causes Collapse of Intermediate Filament Networks with Alteration of Cell Shape and Nuclear Integrity

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**Filaggrin is an intermediate filament-associated protein (IFAP) that aggregates epidermal keratin filaments *in vitro* and is thought to perform a similar function during terminal differentiation *in vivo*. To test this function in living cells, we transiently expressed constructs encoding human filaggrin in both simple epithelial cells (COS-7) and rat keratinocytes. Scanning laser confocal microscopy showed that filaggrin-positive cells had collapsed keratin and vimentin intermediate filament (IF) networks, and that filaggrin partially co-localized with the IF networks. Filaggrin was also detected diffusely in the cytoplasm and nucleus. In contrast, when profilaggrin-like constructs, containing five filaggrin domains separated by the linker sequences, were expressed in cultured cells, immunoreactive granules formed. This finding is reminiscent of the insoluble nature of native profilaggrin that accumulates in keratohyalin granules *in vivo*, suggesting that the linker peptides (present in profilaggrin but not filaggrin) are important for granule formation. Cells expressing filaggrin also displayed disruption of the nucleus and the nuclear envelope; they rounded up and lost attachment to the substratum, in contrast to control cells over-expressing β-galactosidase. This functional test of filaggrin in living cells supports its role in the reorganization and packing of keratin IF in epidermal differentiation. Moreover, the observed effects on cell morphology and nuclear integrity suggest that filaggrin may contribute to the form of apoptosis associated with terminal differentiation in epidermis.** *Key words: intermediate filament-associated protein/ epidermis/apoptosis.*
*J Invest Dermatol 108:179–187, 1997*

Intermediate filament associated proteins (IFAP) are a diverse group of structural proteins that play a role in the macromolecular organization and function of intermediate filaments (IF), an important component of the cytoskeleton (reviewed by Albers and Fuchs, 1992). An IFAP may co-distribute with IFs in cells or be present at IF anchorage sites; it may co-purify with IFs and affect filament organization, assembly and/or macromolecular structure (for reviews, see Foisner and Wiche, 1991; Yang *et al*, 1993; Presland, 1996). Filaggrin, a cationic protein of mammalian epidermis, was one of the first IFAPs to be characterized biochemically and functionally. It aggregates with keratin filaments, as well as other types of IFs, leading to the formation of highly organized bundles or macrofibrils; it was subsequently named filaggrin for this *fil*ament *aggr*egat*in*g activity (Dale *et al*, 1978; Steinert *et al*, 1981). Filaggrin was proposed to function as the interfilamentous matrix protein of the stratum corneum, based on its ability to aggregate keratin IFs *in vitro* into macrofibrils that fill the anucleated cells of the stratum corneum (Dale *et al*, 1978).

Filaggrin is synthesized in the granular cell layer of epidermis as a large, insoluble, and highly phosphorylated precursor, profilaggrin, which is localized in keratohyalin granules and forms the bulk of their content (Steven *et al*, 1990; Manabe *et al*, 1991). Profilaggrin consists of multiple filaggrin repeats flanked by unique N- and C-terminal domains (Haydock and Dale, 1986; Gan *et al*, 1990; Presland *et al*, 1992). Each filaggrin repeat consists of a cationic sequence with a short hydrophobic linker peptide. During terminal differentiation, profilaggrin is dephosphorylated and proteolytically processed, resulting in the removal of these hydrophobic linker peptides, as well as the flanking N- and C-terminal domains, to generate mature filaggrin (Resing *et al*, 1989; 1993a; 1993b; reviewed in Dale *et al*, 1994).

Evidence supporting the function of filaggrin *in vivo* includes the observations that filaggrin (but not profilaggrin) is capable of aggregating keratin IFs (Lonsdale-Eccles *et al*, 1982; Harding and Scott, 1983); that filaggrin is expressed only in keratinizing epithelia (Smith and Dale, 1986), and that premature expression of filaggrin is effectively prevented in living cell layers of the epidermis

Manuscript received July 29, 1996; revised September 17, 1996; accepted for publication October 11, 1996.

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This work was presented in part at the Society of Investigative Dermatology meetings in 1995 and 1996.

Abbreviations: IF, intermediate filament; IFAP, intermediate filament-associated protein; REKs, rat epidermal keratinocytes; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

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by expression as an inactive precursor that subsequently undergoes extensive processing (reviewed in Dale *et al*, 1994). Immunoelectron microscopy of epidermis also suggests that filaggrin is an IF-bundling protein *in vivo*. Hence, the tight packing of keratin IFs into macrofibrils coincides with the dissolution of keratohyalin granules, which occurs with the processing of profilaggrin to form filaggrin. The association of filaggrin with keratins may facilitate disulfide bond formation between keratin polypeptide chains and enable them to survive the massive remodeling that ensues with terminal differentiation. Subsequent chemical modification and degradation of filaggrin, i.e., the loss of filaggrin immunoreactivity in the upper stratum corneum, coincides with the "loosening" of keratin IFs (Manabe *et al*, 1991) and the degradation of filaggrin to free amino acids, which are thought to bind water in the upper stratum corneum (Rawlings *et al*, 1994).

The association of filaggrin with keratin IFs to form macrofibrils has been demonstrated *in vitro* using purified filaggrin (Dale *et al*, 1978; Steinert *et al*, 1981) and filaggrin-like peptides (Mack *et al*, 1993). Nevertheless, the role of the protein in filament aggregation in terminal differentiation of epidermal cells *in vivo* is still controversial. This is based on its poor expression in some pathologic conditions (Manabe *et al*, 1991) and on its possible alternate or additional functions (Rawlings *et al*, 1994; Steinert and Marekov, 1995). The reorganization of the keratin IF network in the transition from the granular to cornified cell layers could result from multiple factors, but the timing of profilaggrin processing makes filaggrin a strong candidate to function as an IFAP *in vivo*. Thus, it was important to demonstrate that filaggrin functions in living cells and is capable of leading to alteration of the cytoskeleton in cells in which it is expressed.

In the current study, we sought to determine whether filaggrin functions as an IFAP using transient transfection as a means of ectopic protein expression in two types of cultured epithelial cells and by examining its effect on the organization of the IF cytoskeleton. We have also investigated the role of the linker peptide in modifying the action of filaggrin in cells by using a profilaggrin-like form of the protein. We report here that expression of human filaggrin leads to the collapse of both keratin and vimentin IF networks in COS-7 cells and of keratin IFs in epidermal keratinocytes. Further, we show that cells expressing filaggrin have a disrupted nuclear envelope. We also demonstrate that expression of profilaggrin-like constructs that include linker peptides yield an apparently less soluble protein, which forms granules within the cells.

## MATERIALS AND METHODS

**Preparation of Filaggrin Constructs** Human filaggrin constructs were prepared both by a polymerase chain reaction (PCR)-based approach and by the direct cloning of human filaggrin repeats into an expression vector containing an *N*-terminal FLAG sequence, which included an ATG initiation codon **(Fig 1)**. Expression vectors containing either the cytomegalovirus (CMV) immediate early promoter (pcDNA3, Invitrogen, San Diego, CA) or the involucrin promoter (H3700-pL2), a generous gift of Dr. J. Carroll (Imperial Cancer Research Fund, London, U. K.), were used. The involucrin promoter plasmid drives high levels of β-galactosidase expression in keratinocytes (Carroll and Taichman, 1992; Carroll *et al*, 1993), whereas the CMV promoter gave high levels of expression in COS-7 cells and variable expression in a rat epidermal keratinocyte cell line (REKs).

*Construction of FG-L and FG+L* Two PCR-derived constructs were prepared either with (FG+L) or without (FG-L) the linker sequence that flanks each filaggrin domain in profilaggrin **(Fig 1)**. For this study, the linker sequence was defined as the peptide RSGRSGSFLY, which corresponds closely with the boundaries of the linker recently defined by mass spectrometry (Thulin and Walsh, 1995). PCR was performed using the oligonucleotides 5′-CCCGCGGCCGC*ACCATG*CAGGTGAGCACTCATGAACAGTCTGAG-3′ (−linker) and 5′-CCCGCGGCCGC*ACCAT*GAGGTCTGGACGTTCAGGGTCTTTCCTC-3′ (+linker); the common downstream oligonucleotide consisted of 5′-CCCGCGGCCGC*TTA*TCCCCCTGACCGGTCACGTGCGGACTC-3′. The oligonucleotides include *Not*I restriction sites for cloning (underlined) and artificial translation initiation and termination codons (italicized). PCR was performed for 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C in a Amplitron I thermocycler (Barnstead-Thermolyne, Dubuque, IA) using the profilaggrin genomic clone cosmid 28.1 as template (Presland *et al*, 1992). The PCR product (~1 kb) was gel purified, digested with *Not*I, and cloned into pcDNA3, or into H3700-pL2 after removal of the β-galactosidase gene with *Not*I. The FG-L construct encodes the mature filaggrin protein (314 amino acids), whereas the FG+L construct encodes a single filaggrin (324 amino acids) domain with a linker at the *N*-terminus, similar to what would be expected during *in vivo* processing.

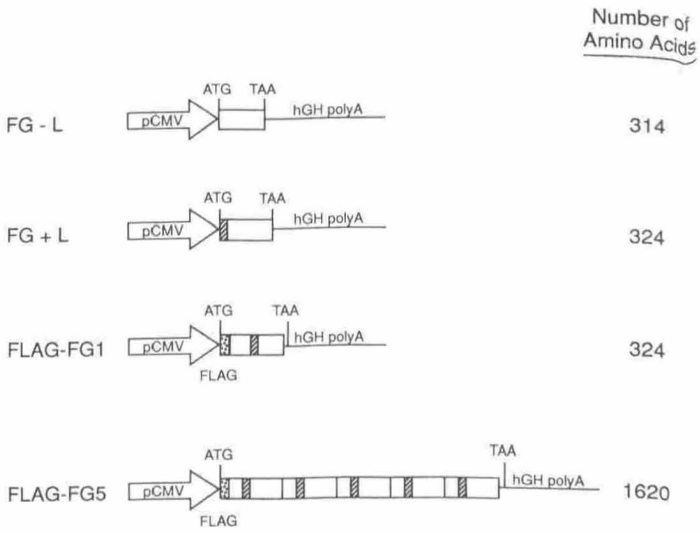


**Figure 1. Human filaggrin expression constructs.** □ indicates sequence of mature form of filaggrin; ▨ indicates the linker sequence; ▩ indicates the FLAG epitope. The FG-L and FG+L constructs were also prepared utilizing the involucrin promoter for studies in REKs. FLAG-FG1 and FLAG-FG5 were detectable with antibodies to human filaggrin and antibodies to the FLAG epitope. Analogous constructs were prepared with filaggrin in the anti-sense orientation. Number of amino acids indicated does not include the FLAG epitope (eight residues).

*Construction of FLAG-FG 1 and FLAG-FG 5* The pcDNA3-FLAG mammalian expression vector was prepared by cloning the multiple cloning site and surrounding sequences of pFLAG-2 (IBI Kodak, New Haven, CT) into pcDNA3. This was accomplished by PCR using primers flanked by *Kpn*I and *Apa*I sites to facilitate direct cloning of the pFLAG-2 sequence into pcDNA3. The sequence of the resulting pcDNA3-FLAG polylinker was verified by DNA sequencing; the hybrid vector contained an *N*-terminal FLAG sequence including an initiation codon, the multiple cloning site from pFLAG-2, and downstream stop codons in all three reading frames.

The one- and five-filaggrin domain constructs were prepared as follows. Two different plasmids (pHX1 and pHX5) containing single human filaggrin domains from cosmid 28.1 were digested with *Xba*I and cloned into pcDNA3-FLAG. Similarly, cosmid 28.1, which contains the 3′-half of the profilaggrin coding sequence (Presland *et al*, 1992), was digested with *Eco*RI, end filled with the Klenow fragment of DNA polymerase I, and cloned into the *Eco*RV site of pcDNA3-FLAG. The resulting construct, pFLAG-FG5, contained five filaggrin domains. Plasmid DNA was prepared by alkaline lysis and purified using Qiagen 500 columns as directed by the manufacturer (QIAGEN, Chatsworth, CA). All constructs were verified by sequencing. These constructs in which the linker sequence is internal encode proteins similar to profilaggrin *in vivo*.

The expression construct for β-galactosidase was pSV-βgal (Promega, Madison, WI).

**Cell Culture and Transfection Procedures** COS-7 African Green monkey kidney cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were passaged weekly. REKs, a rat epidermal keratinocyte cell line (a generous gift from Dr. Howard Baden, Massachusetts General Hospital, Boston, MA) were grown as previously described (Baden and Kubilus, 1983; Haydock *et al*, 1993). All cell lines were tested to exclude the possibility of *Mycoplasma* contamination.

COS-7 cells and REKs were transfected in 60-mm dishes at 60% confluence using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD) essentially as recommended by the manufacturer. For each

transfection, 2.5 μg of filaggrin DNA and 2 μg of pSV-βgal were mixed with 40 μg lipid reagent and serum-free Dulbecco's modified Eagle's medium per ml and incubated at 37°C for 4 h. After 4 h, an equal volume of 20% Dulbecco's modified Eagle's medium was added, the suspension was incubated overnight at 37°C, and the medium was changed. Approximately 48 h after transfection, the cells were harvested and washed in cold phosphate-buffered saline, and lysates were prepared in 0.25 M Tris(hydroxymethyl)aminomethane/HCl, pH 7.8, and assayed for β-galactosidase activity as described (Sambrook *et al*, 1989). For immunofluorescence, approximately $1–4 \times 10^4$ cells were plated on glass coverslips in 12-well plates and transfected as above. Cells were washed in phosphate-buffered saline, fixed, and permeabilized in methanol:acetone (3:1) for 10 min at −20°C, rinsed again in phosphate-buffered saline, and stored at 4°C until antibody labeling was performed. Filaggrin sense and anti-sense constructs were used in parallel in all transfection experiments conducted.

**Antibodies** The monoclonal (AKH1, Dale *et al*, 1987) and polyclonal antibodies (8959, Fleckman *et al*, 1985) to human filaggrin and the polyclonal antibody to rat profilaggrin/filaggrin (466, Haydock and Dale, 1986; Haydock *et al*, 1993) were developed by our laboratory. The monoclonal antibodies AE1 and AE3, which are specific for acidic and basic subfamilies of keratins, respectively, were a generous gift of Dr. T.-T. Sun, NYU Medical Center, New York (Eichner *et al*, 1984). Other monoclonal and polyclonal antibodies were obtained commercially and included monoclonal antibodies to keratin 8 (used for COS-7 cell studies), vimentin, and β-tubulin (Sigma, St. Louis, MO), lamin B (Novocastra, Newcastle-upon-Tyne, U.K.), and the anti-FLAG antibodies M2 and M5 (IBI Kodak). Polyclonal antibodies included pankeratin (BioGenex, San Ramon, CA) and anti-β-galactosidase antibody (US Biochemicals, Cleveland, OH).

**Gel Electrophoresis and Immunoblot analysis** Cultured cells transfected with filaggrin constructs were extracted with 0.25 M Tris(hydroxymethyl)aminomethane/HCl, pH 7.8, in the absence of detergent or urea, and proteins were separated on 7.5%–12% SDS-PAGE gels; Tris(hydroxymethyl)aminomethane/urea extracts of human foreskin epidermis that contain profilaggrin and filaggrin were used as controls (Laemmli, 1970; Sybert *et al*, 1985). Proteins were blotted to nitrocellulose and immunoreactive proteins visualized as previously described with the avidin-biotin method using 4-chloro-1-naphthol (Towbin *et al*, 1979; Haydock *et al*, 1993) or enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). ECL blots were developed on ECL Hyperfilm (Amersham).

**Immunofluorescence Detection of Expressed Proteins and Other Cellular Constituents** For double immunofluorescence, the first primary antibody (monoclonal) was incubated for 1 h and detected with a secondary biotin- labeled horse anti-mouse IgG (Vector Laboratories, Burlingame, CA; 1:400), and streptavidin Texas Red (Vector, 1:800) with multiple rinses between incubations. The second primary (polyclonal) was incubated for 1 h, then rinsed and detected with fluoroscein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Vector, 1:200). Samples were coverslipped using Vectashield mounting medium to minimize fading. Image acquisition was on a Bio-Rad (Richmond, CA) MRC-600 laser scanning confocal microscope equipped with a krypton/argon–mixed gas laser used in dual excitation mode passing 488 nm wavelength for FITC excitation and 568 nm wavelength for Texas Red excitation. Z-step thickness was 0.8 or 1 micron for 60×; optical section thickness was set to give some overlap between optical slices. Bleed through from the red channel into the green channel was never observed, but bleed through from green into red was observed occasionally and subtracted using the Bio-Rad COMOS D-Bleed program. Image files were split and taken into Adobe Photoshop to display image in color as side by side or merged slices or maximum projections. Color prints were outputted to a Tektronix dye sublimation printer at 150 dpi. For DNA staining, samples previously stained for filaggrin were incubated in 0.001% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min then washed with phosphate-buffered saline. DAPI stain was observed using a ultraviolet filter by epifluorescence on an Olympus BH-2 microscope.

## RESULTS

**Single and Multiple-Domain Filaggrin Polypeptides Can Be Expressed in COS-7 Cells and Epidermal Keratinocytes** The filaggrin expression constructs used in this study are shown in **Fig 1**. The FG+L and FG-L constructs produced immunoreactive filaggrin protein when expressed transiently in either COS-7 cells or REKs; the expressed protein was similar in size to authentic human filaggrin **(Fig 2*A*)**. The FLAG-FG constructs containing either one- or five-filaggrin domains also produced proteins of the predicted molecular mass (38 kDa and ~190 kDa, respectively, **Fig 2*B,C***). Proteolytic processing of the five-domain protein was not evident by immunoblot analysis under the growth conditions used (subconfluent cultures were used to optimize immunofluorescence observations). Processing was anticipated in the REK line but was not observed here, perhaps because these cells were not sufficiently differentiated to express the required enzymes or because of species-specific aspects of processing (Resing *et al*, 1989; 1993a).

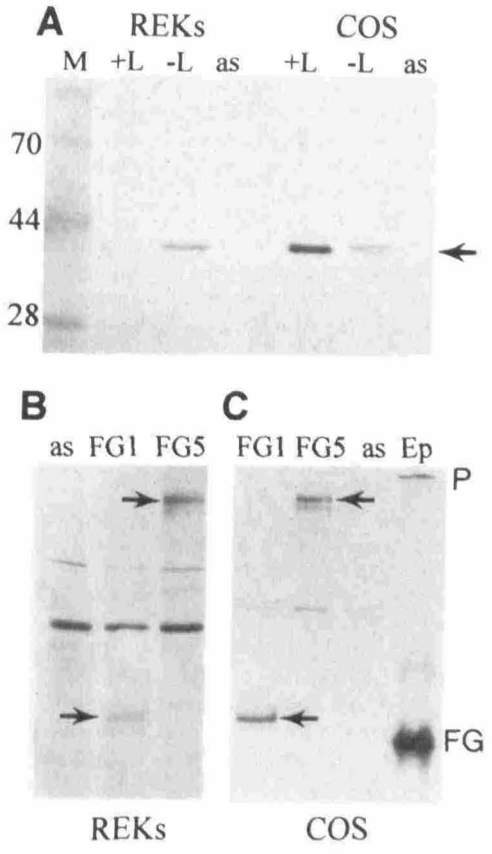


**Figure 2. Ectopic expression of filaggrin in cultured COS-7 cells and REKs analyzed by immunoblot analysis.** *(A)* Transfection with FG-L, FG+L, and FG(as) showing expression of the 37-kDa protein in both cell types, but not in the anti-sense control (as). Immunoblot detection was by avidin-biotin peroxidase complex. Note that the size of the linker is too short to alter migration, and that the expression of FG+L in REKs was weak and variable.*(B)* and *(C)* Transfection with FLAG-FG1, FLAG-FG5, and FLAG-FG(as) in REKs *(B)* and COS-7 cells *(C)* showing expression of one-filaggrin (38 kDa) and five-filaggrin domain constructs (190 kDa, doublet)(→). Control foreskin extract shows native profilaggrin (P) and filaggrin (FG). Immunodetection using polyclonal antibody to human filaggrin and ECL; identical results were obtained with antibody M5 specific for the FLAG epitope (not shown). The FLAG-FG1 protein migrates more slowly than native filaggrin due to the addition of the epitope. Other bands (also observed in the anti-sense controls) are nonspecific.

No filaggrin expression was detected using filaggrin anti-sense constructs **(Fig 2)**. Human filaggrin was not detectable in untransfected COS-7 cells or in REKs. Although REKs express rat profilaggrin (~800 kDa) and filaggrin (42 kDa) after confluence (Haydock *et al*, 1993), the rat protein does not cross-react with the human filaggrin antibody used to measure expression in transfected cells (data not shown). The expression of filaggrin was detectable at all times between 12 and 72 h but was maximal at 48 h in COS-7 cells and 72 h in REKs (data not shown).

**Expression of Filaggrin in COS-7 Cells Results in Collapse of IF Networks** Analysis of cells transfected with the FG-L and FG+L constructs by scanning laser confocal microscopy revealed a

striking reorganization of both vimentin **(Fig 3*A-C*)** and keratin **(Fig 3*D-I*)** IF networks, often into perinuclear bundles or coils in contrast to the dispersed IF network in control cells **(Fig 3*J-L*)**. In all filaggrin-positive cells examined, the IF distribution was altered; this was never observed in control cells. The filaggrin staining was diffuse throughout the cytoplasm and nucleus, but staining co-localized at least partially with vimentin or keratin IFs (e.g., yellow/orange double immunofluorescence as in **Fig. 3*C,F,I*)**. In COS-7 cells, the proteins encoded by both filaggrin constructs appeared to collapse the IF networks (compare **Fig 3*B*** and **3*H*** to normal vimentin and keratin networks shown in anti-sense controls, **Fig. 3*K,L*)**. These results suggest that filaggrin can function as an IFAP (or IF-bundling protein) in living cells. On the other hand, expression of filaggrin in COS-7 cells and REKs did not collapse microtubules into perinuclear coils (data not shown), suggesting that the major effect of filaggrin was specific for the IF cytoskeleton.

**The Linker Peptide Alters Filaggrin Function and Distribution in COS-7 Cells and REKs** The linker sequence is present between each filaggrin domain in profilaggrin and is removed by proteolysis during processing and formation of mature filaggrin (reviewed in Dale *et al*, 1994). Two different types of constructs were used to test the effect of the linker peptide sequence on IF association and cell function; (i) a filaggrin domain with a linker sequence at the *N*-terminus (FG+L) and (ii) FLAG-tagged constructs with the linker sequence in an internal location with either one (FLAG-FG1) or five complete filaggrin domains (FLAG-FG5) (see **Fig 1**). The latter two constructs are more like profilaggrin *in situ*, whereas the FG+L construct, with the linker at the *N*-terminus, would be present only briefly during processing of profilaggrin to filaggrin in transitional epidermal cells.

Expression of profilaggrin-like constructs that contain one or more linker peptide sequences resulted in formation of protein aggregates of either round or irregular shape in REK cells **(Fig 4*B,D*)** in contrast to the FG-L construct that lacked linker peptide(s) and was diffusely localized **(Fig 4*A*)**. Similar granular aggregates were observed in COS cells **(Fig 4*F,I*)**. In both cell types the immunoreactive granules varied in size and were present in the cytoplasm and nucleus. They are reminiscent of the immunoreactive (profilaggrin-containing) granules in differentiating REKs **(Fig 4*C*)** (also Haydock *et al*, 1993). The granules detected in transfected cells, however, also reacted with a monoclonal antibody specific for the FLAG-epitope **(Fig 4*K*)**, verifying their ectopic origin.

Two types of evidence indicate that the expression of immunoreactive granules was specific for the filaggrin linker sequence and not the FLAG peptide. First, the FLAG-FG(as) construct, which encodes a protein of 26 amino acids in length (including the FLAG peptide), never yielded a granular staining pattern. Indeed, many investigators have used this hydrophilic epitope with no reports of a granular immunofluorescence pattern of FLAG-tagged protein (see for example, Kouklis *et al*, 1994). Second, expression of the FG+L construct, which lacks the epitope tag, also yielded a granular immunofluorescence pattern in REKs **(Fig 4*B*)**. Constructs with the linker internal to filaggrin sequences (the structure most similar to native profilaggrin) always yielded the granular pattern of staining. Intranuclear localization of filaggrin (but not keratins) was observed in varying degrees in these cells and other cells expressing all of the constructs tested (see section below).

Transfected cells expressing the profilaggrin-like constructs also showed evidence of collapse of IF networks. Keratin filaments were collapsed around the nucleus of positive REK cells **(Fig 4*E*, *arrow*)**. COS-7 cells expressing FLAG-FG1 display various degrees of IF disruption ranging from partial IF aggregation **(Fig 4*G,H* *arrow*)** to total disruption in highly positive cells (note cell 3 in **Fig 4*I,J*)**.

**Filaggrin Expression Results in Changes Associated with Cell Death** As described above, cells expressing filaggrin, particularly the mature form, showed aggregation and collapse of their IF networks. This finding was consistent with the predicted function of filaggrin. A somewhat surprising finding of these studies, however, was that cells expressing filaggrin displayed changes in shape and nuclear integrity. In COS-7 cells expressing filaggrin, the cell cytoplasm was condensed; cells were frequently rounded up and seemed to be losing adherence to the substratum **(Fig 5*A,B*)**. The change in cell shape was observed in cells expressing filaggrin constructs with and without the FLAG epitope tag (compare **Fig 4*F,I*** to **Fig 5*A,B*)**.

Cells expressing filaggrin also showed evidence of breakdown of the nuclear compartment. Both β-galactosidase and filaggrin were detectable in the nucleus of filaggrin-positive cells **(Fig 5*A,B,D*;** see also **Fig. 3*A*, 4*D*)**; in contrast, β-galactosidase was very rarely (if ever) observed in the nucleus of COS-7 cells transfected with the β-galactosidase plasmid and/or the anti-sense filaggrin construct **(Fig 5*C*,** and not shown), demonstrating that nuclear disruption is not a nonspecific result of transfection.

The presence of β-galactosidase and filaggrin in the nucleus of cells expressing filaggrin suggested a disruption of the nuclear envelope in these cells. To further explore this phenomenon, transfected COS-7 cells were stained with antibodies to human filaggrin and nuclear lamin B **(Fig 6)**. In the most extreme cases, the nuclear envelope of filaggrin-positive cells was disassembled into star-shaped aggregates that reacted with the lamin antibody. This was observed with both the FLAG-epitope–tagged constructs (*arrows* in **Fig 6*B***) and the FG+L and FG-L constructs (data not shown). In some cells, nuclei were condensed or irregular in shape **(Fig 6*F*, *arrow*)**, whereas in others filaggrin-reactive granules lined up along the nuclear envelope **(Fig 6*C,D*)**. COS-7 cells transfected with an anti-sense construct **(Fig 6*G,H*)** showed typical nuclear envelope staining with some punctate staining within the nucleus.

Morphologic evidence of nuclear condensation was also observed in cells expressing filaggrin constructs. Transfected cultures were stained for DNA with DAPI; filaggrin-expressing cells frequently had condensed and dysmorphic nuclei **(Fig 7*A,B*, *arrows*)**. Analysis of multiple transfections showed that 49% (315/648) of filaggrin-positive cells had condensed or abnormal nuclei compared to 11% (637/5683) of cells transfected with a control anti-sense construct ($p < 0.001$) **(Fig 7*C*)**. There was no significant difference in the percentage of condensed nuclei in cells transfected with different filaggrin sense constructs.

## DISCUSSION

**Expression of Filaggrin Constructs in COS-7 Cells and Epidermal Keratinocytes Results in Collapse of IF Networks** Filaggrin was the first protein characterized for its ability to cause IF aggregation in an *in vitro* assay (Dale *et al*, 1978), and on that basis is now classed with a growing number of IFAPs (reviewed by Presland, 1996). In this work we have tested the hypothesis that filaggrin functions as an IFAP in living epithelial cells and demonstrated that it has profound effects on IF distribution, supporting this hypothesis. Expression of the mature form of filaggrin led to the collapse of the vimentin and keratin IF networks into juxtanuclear aggregates in COS cells **(Fig 3)** and resulted in keratin filament collapse around the nucleus in REKs (e.g., **Fig 4*E***). The results are consistent with the original filaggrin-keratin mixing experiments conducted *in vitro*, which resulted in formation of macrofibril-like structures (Dale *et al*, 1978; Steinert *et al*, 1981). *In vitro* results, however, cannot *a priori* be extended to the *in vivo* situation; therefore, our results are an important extension to demonstrate filaggrin function in living cells. The immunofluorescence technique at the level of light microscopy does not prove direct association of the proteins, and follow-up immunoelectron microscopy studies are needed to verify the direct interaction. Nevertheless, collapse of the IF network was a common feature of filaggrin-positive cells and was rarely observed in filaggrin-negative cells. In addition, Triton extraction of transfected cells showed that a portion of the expressed filaggrin remained in the detergent-insoluble cytoskeletal fraction, indicative of an IFAP (data not shown). Taken together with the *in vitro* mixing experiments using purified proteins, the results strongly support the interaction of

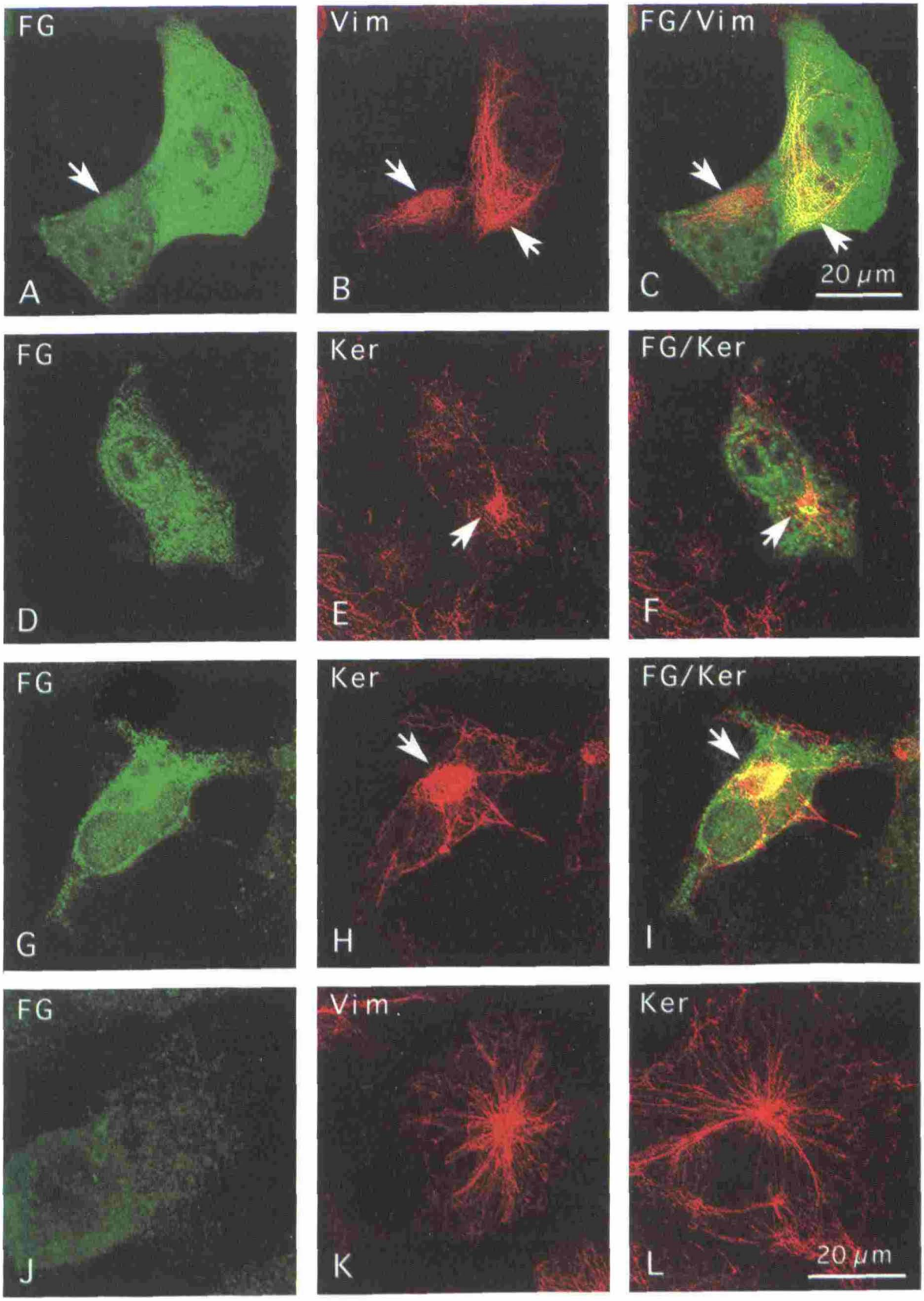


**Figure 3. Filaggrin expression results in collapse of IF networks.** Transfected COS-7 cells were fixed and stained by double immunofluorescence with polyclonal antibody to human filaggrin (FG) and monoclonal antibodies to either vimentin (Vim) or keratin 8 (Ker), as indicated. Identical fields are shown in *(A)* and *(B)*, and the two-color overlay *(C)*, as well as in *(D-F)* and *(G-I)*; *(J)* and *(K)* are the same field, whereas *(L)* is a separate view. *(A-C)* and *(D-F)*, FG-L construct; *(G-I)*, FG+L construct; *(J-L)*, FG(anti-sense) construct. Note collapse of both vimentin and keratin IF networks in contrast to controls *(K and L)*. Filaggrin is co-localized with the collapsed IF (*arrows*, indicated by orange or yellow color depending on the relative signal intensity) as well as diffusely in the cytoplasm and nucleus of positive cells.

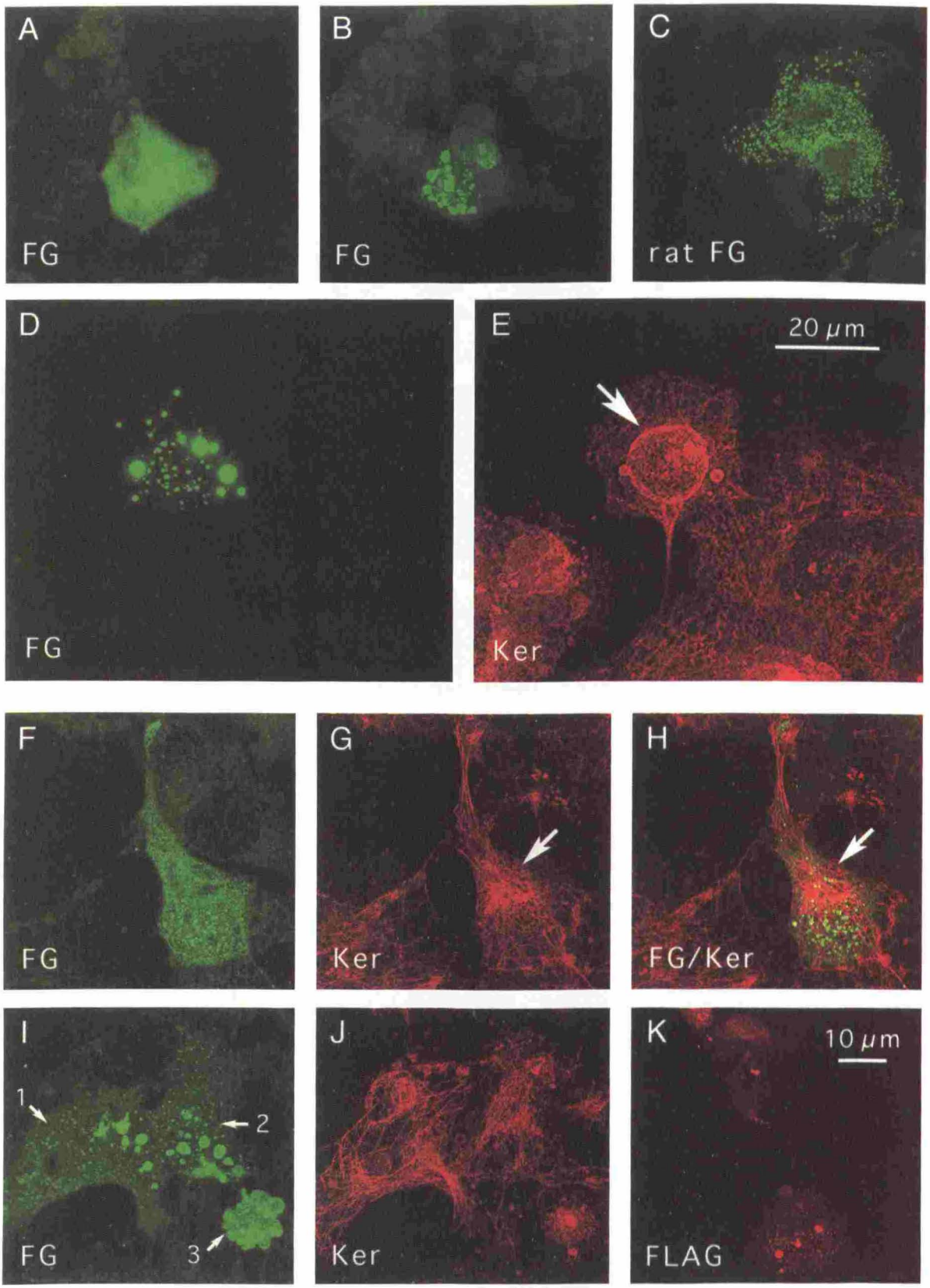


**Figure 4. Expression of profilaggrin-like constructs yields granular immunoreaction product in REKs and COS-7 epithelial cells.** Cells were transfected with constructs containing the linker sequence *(B-K)* or the mature filaggrin construct *(A)*, fixed, and stained for human filaggrin (FG) and keratins (Ker). REKs: *(A)* FG-L; *(B)* FG+L; stained with polyclonal anti-human filaggrin; *(C)* stained with polyclonal anti-rat filaggrin to indicate endogenous profilaggrin in a stratified region of the culture; *(D)* and *(E)* FLAG-FG5, stained using monoclonal anti-human filaggrin and polyclonal anti-pankeratin (the same field is shown). Note that immunoreactive granules observed in cells transfected with profilaggrin-like constructs *(B,D,F,I)* are similar to endogenously expressed rat profilaggrin *(C)*. Also note partial collapse of the REK keratin IF network (*E*, →). COS-7 cells: *(F-J)* transfected with FLAG-FG1; *(K)*, FLAG-FG5. *(F-J)* were stained using polyclonal anti-filaggrin with an FITC-secondary and monoclonal anti-keratin 8 with a Texas Red secondary; *(H)* is the two-color overlay of *(F)* and *(G)*. *(K)* monoclonal antibody M2 to the FLAG epitope (identical results were observed with M5, not shown). Note the partial collapse of the keratin IF network in *(G,H)*; whereas in *(I-J)*, three cells labeled 1, 2, and 3 show increasing expression of FLAG-FG1, with extensive disruption of the keratin IF network in cell 3.

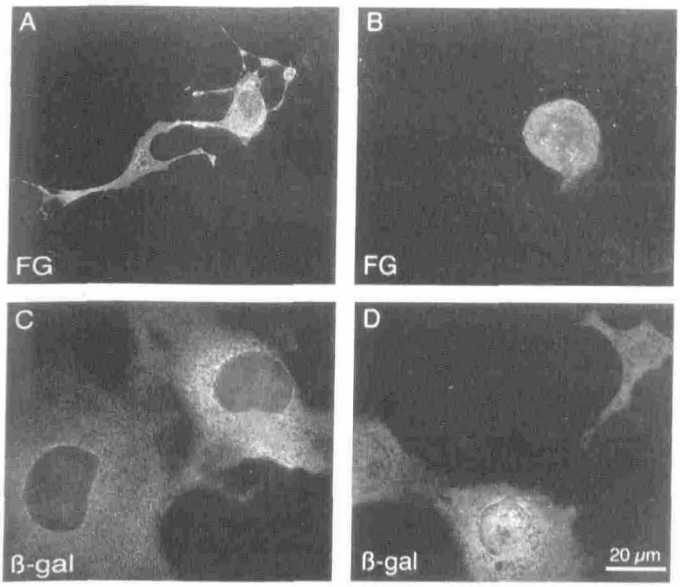


**Figure 5. Filaggrin expression results in alteration of cell shape and loss of attachment to the substratum.** Transfected COS-7 cells, fixed and stained for immunofluorescence with polyclonal antibody to human filaggrin (FG), or polyclonal antibody to β-galactosidase (β-gal). *(A)*, *(B)*, and *(D)* were transfected with the FG-L construct, whereas *(C)* was transfected with the anti-sense (FG(as)); all cultures were cotransfected with the expression construct for β-galactosidase. Expression of the ectopic protein is detected in the nucleus *(A,B,D)* as well as the cytoplasm, whereas only cytoplasmic β-galactosidase expression is detected in *(C)*. Note that the filaggrin-positive cells in *(A)* and *(B)* have compact cytoplasm and altered cell shape and are raised from the surface.

filaggrin and IFs. The filaggrin-mediated alterations did not result in a general disruption of the cytoskeleton, as microtubules were relatively unaffected by filaggrin expression. The degree of IF aggregation or disruption may depend on the particular construct expressed as well as the level of transgene expression; this was also the case for filaggrin and IF association in the *in vitro* studies (Steinert *et al*, 1981).

**Expression of Profilaggrin-Like Constructs Yields Granules** Transient transfection of constructs containing one or more linker peptide sequences yielded immunoreactive aggregates or insoluble protein. This finding was surprising, but suggestive of the native epidermal disposition of profilaggrin in keratohyalin. The results indicate that the linker peptide itself influences the behavior of the expressed protein in the cell. The effect of the linker may be via formation of intermolecular associations with filaggrin sequences or by association between the hydrophobic linker sequences themselves, leading to aggregation. Such an association might be expected to be stronger in the FLAG-FG5 protein (which contains five linkers) than in the FLAG-FG1 protein, which has a single linker. Experimentally, the five-domain protein (FLAG-FG5) yielded only granular staining whereas the FLAG-FG1 construct gave both granular and diffuse staining. *In vitro* studies demonstrate that proteolytic profilaggrin fragments containing three to five filaggrin domains are largely insoluble in aqueous buffers, whereas one- to two-domain fragments partition between the soluble and insoluble fractions (Resing *et al*, 1995b; Thulin, 1995), consistent with our findings.

Profilaggrin is phosphorylated at multiple serine residues (Lonsdale-Eccles *et al*, 1982; Resing *et al*, 1985; 1995a), and phosphate has been suggested as a potentially important factor for the correct folding of profilaggrin and subsequent keratohyalin formation (Dale *et al*, 1994). Hence, an alternative explanation for the aggregates in cells expressing profilaggrin-like constructs is that the protein is phosphorylated. Two-dimensional gel electrophoresis, however, showed that, although some phosphorylation of the ectopically expressed filaggrin and one- and five-domain proteins may occur, it is not a major feature of the filaggrin proteins expressed in cultured cells (data not shown). We conclude that the formation of granules with profilaggrin-like constructs cannot be explained in terms of extensive phosphorylation.

**Ectopic Expression of Filaggrin Results in Nuclear Changes and Apparent Cell Death** A dramatic finding of this study was that filaggrin also produced profound effects on cell shape, which often coincided with disruption of the nuclear membrane and loss of attachment to the culture dish. These effects were observed with both filaggrin and profilaggrin-like constructs. Loss of cell viability in filaggrin-positive cells was shown by the condensation of cytoplasm and nucleus, and lifting of cells from the surface.

Filaggrin toxicity is also shown by expression of both filaggrin and the co-transfected β-galactosidase in the nucleus **(Figs 5; 3*A*)**. In cells expressing only β-galactosidase, the immunoreactive protein was never observed in the nucleus (e.g., **Fig 5*C***), indicating that overexpression of the control protein *alone* was not sufficient to induce changes in nuclear integrity and IF organization. Intranuclear localization of filaggrin, but not IF proteins, was observed to varying extents in cells expressing all of the constructs tested. Filaggrin could disrupt the nuclear membrane integrity directly, by interacting with nuclear lamins, or indirectly, by activating apoptotic pathway(s) as a result of cytoskeletal damage and cellular stress. The nuclear lamina network, which lies on the inner aspect of the nuclear envelope, consists largely of type V lamin IF proteins (Aebi *et al*, 1986; reviewed in Gerace and Burke, 1988). Although filaggrin interacts with multiple IF proteins (Steinert *et al*, 1981), its association with lamins has not been directly evaluated. In this study, co-localization of lamin B and filaggrin in some cells (e.g., **Fig 6*C,D***) suggests that filaggrin can associate with nuclear lamins (as well as with the cytoplasmic IF network). This is consistent with the observation that filaggrin interacts with the conserved α-helical rod domain present in all IF proteins, including nuclear lamins (Mack *et al*, 1993). Nevertheless, filaggrin did not co-localize with depolymerized lamins when the nuclear envelope was dispersed **(Fig 6*B*)**.

An alternative explanation for nuclear changes is that filaggrin indirectly causes the disruption of the nuclear envelope by activation of the apoptotic pathway as a result of a damaged cytoskeleton. The cell morphology, including shrinkage of the cytoplasm around the nucleus and nuclear condensation, is consistent with the occurrence of apoptosis (for reviews, see Cohen, 1991; Haake and Polakowska, 1993). Nuclear envelope disruption also occurs in apoptotic cells, coinciding with degradation of nuclear lamins to lower molecular weight peptides (Oberhammer *et al*, 1994; Neamati *et al*, 1995).

Our findings show some similarities to other studies in which genes encoding mammalian and virus-encoded IFAPs, or specific domains of IFAPs, were ectopically expressed in cultured epithelial cells in order to study their function (reviewed in Presland, 1996). Both desmoplakin I and the related protein, plectin, contain C-terminal sequences consisting of repeating domains of 176 amino acids that co-align with, and eventually collapse, the keratin and vimentin IF networks of epithelial cells into perinuclear aggregates (Stappenbeck and Green, 1992; Stappenbeck *et al*, 1993; Wiche *et al*, 1993). Collapse of the IF cytoskeleton also has been reported for novel IF-associated (or disrupting) proteins encoded by several DNA tumor viruses, including the E4 protein of certain human papillomaviruses (Doorbar *et al*, 1991; Roberts *et al*, 1993; 1994). Filaggrin, however, had additional effects on cell shape, nuclear membrane structure, and apparent cell viability, suggesting a toxic effect. Although constructs encoding certain plectin (Wiche *et al*, 1993), E4 (Roberts *et al*, 1993; 1994), and tailless cytokeratin proteins (Bader *et al*, 1991) were localized to varying degrees in the nucleus, there was no apparent effect on nuclear membrane integrity or cell viability. The question remains: does filaggrin cause IF collapse, which then results in the loss of nuclear integrity and localization of filaggrin in the nucleus or is filaggrin toxic to the cell leading to nuclear changes and subsequent IF collapse? The first mechanism is supported by our results showing IF collapse using FG-L constructs, as well as by the original *in vitro* aggregation

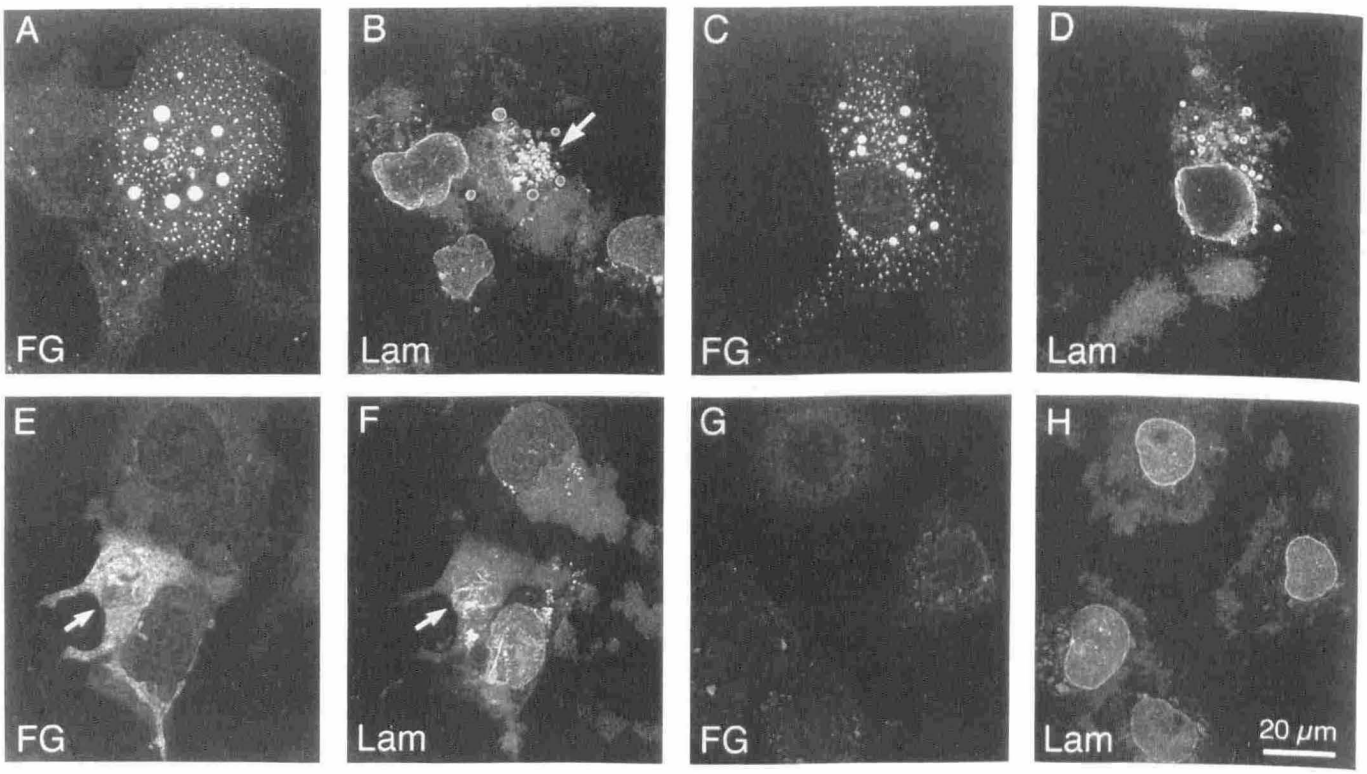


**Figure 6. Filaggrin expression disrupts the nuclear envelope.** Transfected COS-7 cells, fixed and labeled for double immunofluorescence with polyclonal anti-human filaggrin (FG) with an FITC secondary and monoclonal antibody to nuclear lamin B with a Texas Red secondary *(A-H)* and observed by confocal microscopy. The pairs ((*A*) and *(B)*, etc.) represent two views of the same field. *(A-D)* were transfected with the FLAG-FG5 construct; *(E)* and *(F)* with the FG-L construct; and *(G)* and *(H)* with the anti-sense (FLAG-FG(as)) construct. Note loss of the nuclear envelope in filaggrin-positive cells in *(B)* in which star-shaped aggregates are stained with the lamin antibody (→). The cell in *(D)* shows co-localization of filaggrin-positive granules with the nuclear envelope. The cell is *(F)* shows a collapsed nucleus (→). Normal lamin staining is seen in the anti-sense control *(H)*.

studies; a toxic effect of filaggrin cannot be ruled out at this time, however, and may be one effect of the profilaggrin-like granules expressed in the cytoplasm and nucleus (see **Fig 4**).

**Results of Expression of Filaggrin by Transient Transfection Parallel Events of Terminal Differentiation in Keratinocytes** During the transition of the epidermal granular cell to the cornified cell, the profilaggrin in keratohyalin granules is converted enzymatically to filaggrin, which interacts with keratin IFs. Keratin IFs then become densely packed in the condensed cytoplasm characteristic of the stratum corneum. Our results offer strong support for the hypothesis that filaggrin aids in the dense packing of keratin IFs in the cornified cells and suggests two additional important characteristics of filaggrin and profilaggrin. First, that the linker peptide sequence contributes to profilaggrin aggregation into granules, and therefore may be critical in preventing premature IF aggregation, and second, that filaggrin (perhaps in combination with other proteins) may be involved in triggering cell death as it occurs during the terminal differentiation of keratinocytes. It has been suggested that terminal differentiation in epidermis constitutes a special form of apoptosis or programmed cell death (Fesus *et al*, 1991; Haake and Polakowska, 1993). Many events occur at this transition to yield the protective, functional outer layer of the skin, including the loss of cellular organelles and the nucleus, and degradation of cellular DNA. Cells in transition between the granular and cornified layers have been shown to stain for fragmented DNA characteristic of apoptosis (Gavrieli *et al*, 1992; Polakowska *et al*, 1994; Tamada *et al*, 1994). In this study, transfected cells expressing filaggrin show collapse of cell cytoplasm, disruption of the nuclear envelope, nuclear condensation, rounding up of cells, and loss of attachment, all features associated with programmed cell death.

Our results support the role of filaggrin as an IFAP in living cells and show that it leads to reorganization of both vimentin and keratin IF networks. This functional test of the action of filaggrin

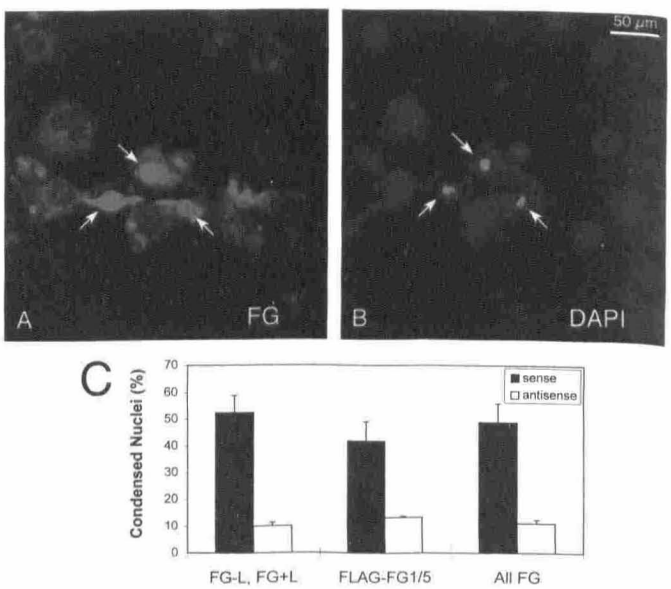


**Figure 7. Filaggrin expression results in alterations in nuclear chromatin.** COS-7 cells transfected with FLAG-FG1 and stained with polyclonal anti-human filaggrin (FG) with an FITC secondary *(A)* and with DAPI stain for DNA *(B)* and observed by epifluorescence. Note the condensed nuclei in the filaggrin positive cells (→). *(C)* Summary of analysis of DAPI-stained nuclei of COS cells expressing filaggrin. The percentage of abnormal or condensed nuclei is shown for cells expressing the two types of filaggrin constructs, compared to cells transfected with a control (filaggrin anti-sense) construct. The results summarize data from several transfection experiments. *Bars* indicate ± 1 SD. In each data set, the difference between sense and anti-sense constructs is highly significant ($p \leq 0.001$).

gives strong support for its role in the packing of keratin IFs in the differentiating cells of the epidermis. Expression of profilaggrin-like constructs containing linker sequences yields protein aggregates, while filaggrin itself is detected throughout the cell; both cause IF network collapse and result in changes of cell shape and loss of nuclear integrity, all features that occur during terminal differentiation in keratinizing epithelial tissues.

*We wish to thank Dr. Joseph Carroll for the involucrin promoter plasmid, Janet Kimball for excellent technical assistance, Barbara Hager for assistance with cell culture, Dr. Karen Stephens and Ms. Lynda Le of the Molecular Genetics Core Laboratory for DNA sequencing, and Dr. William Carter for critically reading this manuscript. We gratefully acknowledge the W.M. Keck Center for Advanced Studies for use of confocal microscopy facilities. This work was supported by National Institutes of Health Grants R37 DE-04660 and P01 AM-21557 (to B. A. Dale). R. B. Presland is the recipient of a Career Development Award from the Dermatology Foundation.*

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